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Selection of *Brassica napus* L. embryogenic microspores by flow sorting

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ABSTRACT

Flow cytometry can be used to select and sort microspore subpopulations of Brassica napus cv. Topas. Data obtained from embryogenic microspore populations were used to identify potentially embryogenic microspores from developmentally heterogeneous microspore populations based on differences in forward light scatter and green autofluorescence. Culture enrichment for embryogenic microspores is possible. Frequencies of 8 and 14% microspore embryogenesis were obtained when selected 16 h and 72 h after culture initiation. This represents 5- and 13-fold increase in microspore embryogenesis compared to non-sorted controls.

INTRODUCTION

Flow cytometry has a wide range of applications in studies of cell population dynamics and characteristics. Advances in plant regeneration from single cell suspensions enhanced the value of applying flow cytometry to sort plant protoplasts, select for heterokaryon products from protoplast fusion experiments and subsequently regenerate plants (Redenbaugh et al. 1982; Alexander et al. 1985; Afonso et al. 1985; Glimelius et al. 1986; Pauls and Chuong 1987). Flow cytometry could also be useful for separation of haploid and diploid embryogenic plant cell populations based on nuclear DNA content (Arndt-Jovin and Jovin 1977). Moreover, transformation products from cocultivation or direct DNA uptake experiments could be quickly identified and selected if fluorescence markers were built into the vectors. Factors governing the flow cytometric analysis and sorting of plant cells have been investigated in detail by Harkins and Galbraith (1987).

Recent advances in Brassica napus microspore culture (Keller et al. 1988; Pechan and Keller 1988; Fan et al. 1988) allow us to examine the usefulness of flow cytometry for microspore culture manipulation. As the first step, it is important to ascertain whether and how efficiently flow sorting can differentiate between microspore subpopulations and select for microspores capable of undergoing embryogenesis.

MATERIALS AND METHODS

1. Microspore culture

Plants of Brassica napus cv. Topas were grown at 10/5°C day/night temperature, 16-h photoperiod, 300 $\mu E\ m^{-2}s^{-1}$ light intensity and 50% relative humidity. Sterilized 3-5 mm buds were placed in a modified B_5 medium as described by Keller and Armstrong (1977) but free of growth regulators and supplemented with 13% sucrose, macerated with a glass rod, released microspores filtered through a 44 µm Nitex screen and washed 3x by centrifugation in the B_5 -13% sucrose medium at 100 g for 3 min. Microspores were subsequently suspended in a modified Lichter medium (Lichter 1982), without growth regulators or potato extract, plated onto 60 x 15 mm (Falcon 1007) petri dishes and incubated in darkness at 32.5°C. Prior to cell sorting, microspores were pelleted at 200 g for 3 minutes and resuspended in a modified Lichter medium to a final concentration of 0.5 to 1.0 x 10^6 microspores per ml. Microspores were sorted at 16 h and 72 h after culture initiation.

2. Flow cytometry

a) Method for analysis:
Flow cytometry was performed utilizing an EPICS C (Coulter Electronics, Hialeah, FL) instrument with a Coherent (Palo Alto, CA) 5-watt Innova 90 argon-ion laser tuned to 488 nm at 500mW. Green fluorescence was analyzed using a 525 nm interference filter and a 550 nm SP dichroic mirror in optical block two. In block one, 488 nm dichroic and a 575 nm interference filters were installed. The two additional filters utilized were a 515 nm long-pass and a 525 nm interference filters in optical blocks one and two, respectively. The signals were processed with a log-linear amplifier. Data analysis was performed with Coulter EASY 88 Data Management System (Coulter Electronics, Hialeah, FL). QUADSTAT program (version 2.1) was used for dual parameter light scatter data analysis.

b) Method for sorting:
Highly embryogenic microspore cultures, originating from specifically selected buds (Pechan and Keller 1988), were used to accumulate flow cytometry data on embryogenic microspores for 4 days subsequent to culture initiation. This

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data was entered into the computer memory for subsequent selection of potentially embryogenic microspores from developmentally heterogeneous microspore populations.

Sorting bitmaps were set on logarithmic two parameter histograms of the relative fluorescence intensity versus forward light scatter. A vibrating piezoelectric quartz crystal tuned at the frequency of 32 kHz broke the flow stream into separate droplets as the stream passed the tip of the flow chamber and was just beyond the point of laser interrogation. Sheath fluid pressure differential was 13-15 psi. The flow tip utilized had a 76 µm inner diameter. The hydrodynamics of the system is designed in such a way that every third droplet should contain a microspore. A microspore that met the bitmap specifications received a charge that was either negative or positive. As the droplets passed between the two electrostatically charged plates of the sort deflecting assembly, they were deflected to the respective reversely charged plates (left or right). A 0.8% saline solution was used as the electrolyte medium. At initial concentrations of 1 x 10^6 microspores per ml, the flow unit sorted approximately 300-500 microspores per second. In order to reduce the possibility of atmospheric contamination during sorting two additional procedures were adapted. Prior to sorting the decontamination procedure of the fluidic system by Redenbaugh _et al_. (1982), was followed with some modification. After operating the fluidic system for a minimum of 5 minutes with 70% ethanol, 13% sucrose solution was flushed through for a minimum of 2 minutes to eliminate any osmotic stress. The left and right cell collectors were also modified. Ten mm diameter plastic test tubes were cut to 8 cm heighth with a false bottom. This adapter permitted the collecting glass tube to be within 2.5 cm of the sort deflecting assembly, thus reducing the exposure of the biological material to laboratory atmosphere. It also ensured that microspores fell directly into the modified Lichter medium rather than onto a test tube wall. Although the saline solution was not sterile, only one out of nine experiments was contaminated.

Subsequent to flow sorting, microspores were pelleted by centrifugation (200 g for 3 min), resuspended in 1.25 ml of a modified Lichter medium and plated onto 60 x 15 mm (Falcon 1007) petri dishes. Microspores were incubated in darkness at 32.5°C up to day 4 after culture initiation and subsequently transferred to 25°C. The number of embryos formed were counted 21 days after culture initiation. Where appropriate, samples of microspores and proembryos were stained with DAPI (4',6-diamidino-2-phenylindole, Sigma Co.), a DNA specific stain, for cytological observations (Pechan and Keller 1988). Microspore diameters were measured with an eyepiece micrometer mounted on a Zeiss microscope.

RESULTS AND DISCUSSION

Flow cytometry offers an efficient and quick way to classify and sort a heterogeneous population of microspores into a number of subpopulations (Fig. 1). Embryogenic microspores can be identified and sorted: such microspores are capable of undergoing normal embryogenic development and subsequently form plants. Thus 7.7% of the microspores formed embryos when selected at 16 h after culture initiation; at 72 h after culture initiation, 13.7% formed embryos (Tab. 1). This represents frequencies 5-13x higher than the unsorted controls. Flow sorting can thus

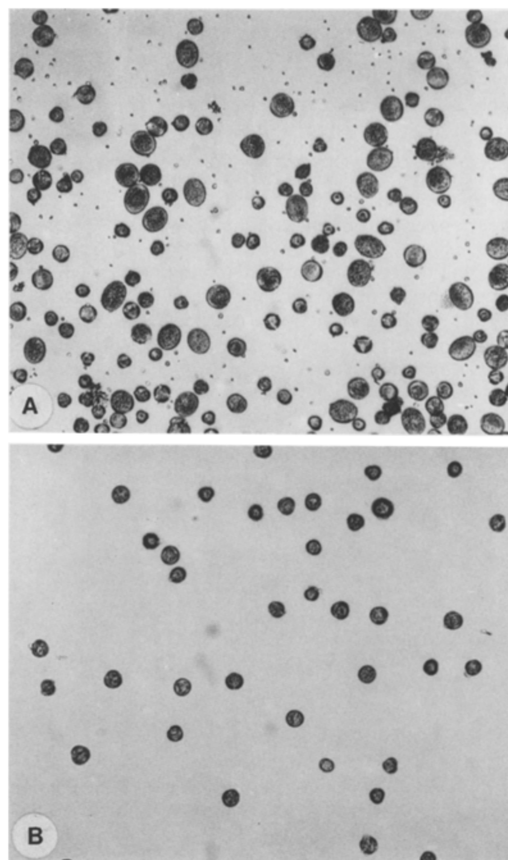

Figure 1. Highly heterogeneous microspore culture prior to (A) and subsequent (B) cell sorting at 16 h after culture initiation. Potentially embryogenic microspores were selected.

Table 1. Percentage embryo formation (means ± SE) for microspores/proembryos sorted 16 h and 72 h after culture initiation. Control (A) = non-sorted microspores/proembryos; control (B) = microspores/proembryos which passed through the flow cytometer but were not sorted. Data are based on 8 experiments.

	Sorted	Control (A)	Control (B)
16 h	7.7 ± 1.76	1.4 ± 0.13	0.3 ± 0.02
72 h	13.7 ± 2.96	1.0 ± 0.19	1.1 ± 0.30

be used to enrich for embryogenic microspores. The frequencies of embryo enrichment are comparable to the percoll gradient method (Fan _et al_. 1988).

Microspore selection based on green autofluorescence and forward light scatter, expressed on a log scale, were the optimal criteria to differentiate microspores into subpopulations. Forward light scatter signal gives an indication of the particle size. The source of green autofluorescence in the microspores is not known. Data expressed on a log vs log display format, rather than a linear display format, presented the best microspore population

separation. Red autofluorescence and 90° light scatter were also tested but deemed unsuitable for identifying embryogenic microspores.

Embryogenic microspores originate from mid to larger sized microspores (20-25 µm diameter) of lower green autofluorescence emission intensities (Figs. 1 and 2). The profile of this subpopulation changed by 72 h after culture initiation: they had grown in size due to nuclear divisions and cell expansion and had also shifted to higher green autofluorescence emission intensity (Fig. 2). Cytological observations revealed the embryogenic subpopulations were intermixed with nonembryogenic mid to late binucleate microspores. Pechan and Keller (1988) have shown such microspores can undergo only one division of the generative nucleus. Thus, culture of binucleate microspores should be avoided in order to improve the ability to identify embryogenic microspores.

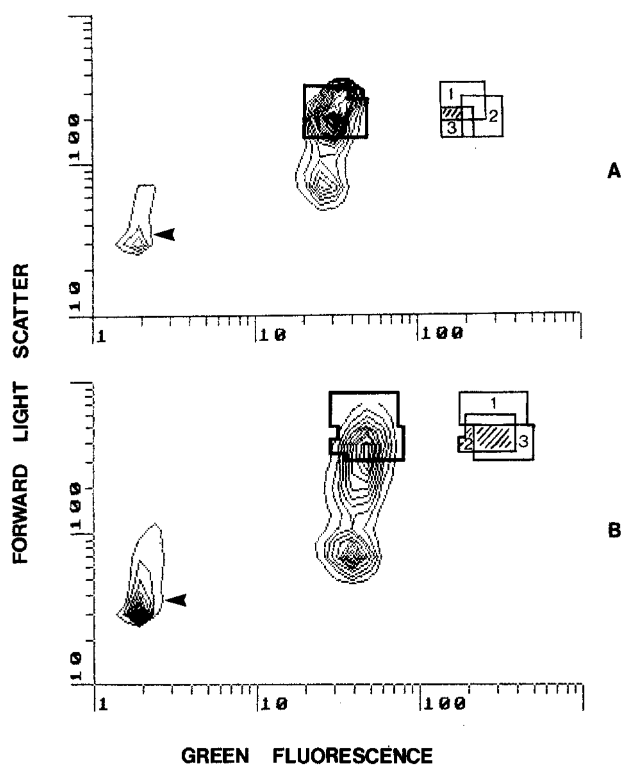

Figure 2. Flow cytometry bitmaps of forward light scatter versus green autofluorescence, plotted on a logarithmic scale, for a heterogeneous microspore culture at 16 h and 96 h after culture initiation. Sorted microspore subpopulations are confined to the areas indicated (1, 2 and 3). These areas correspond to the dark outlines on the bitmaps. Highest embryogenic subpopulations are located in shaded areas.
(A) microspore culture at 16 h. 1 = 10.6% of microspores formed embryos; 2 = 1.2% of microspores formed embryos; 3 = 13.1% of microspores formed embryos. (B) microspore culture at 72 h.
1 = 0.9% of microspores formed embryos; 2 = 18% of microspores formed embryos; 3 = 8% of microspores formed embryos.
Note: Microspores located outside the indicated areas rarely formed embryos. Arrows indicate cell debris.

The percentage of sorted microspores undergoing embryogenic development increases with the age of the culture. One explanation of this observation is that 72 h after culture initiation embryogenic microspores have undergone a number of nuclear divisions and, with the cell wall being actively formed (Pechan unpublished), were likely less susceptible to damage as a result of flow sorting manipulation. Microspores sorted 16 h after culture initiation appeared to be more susceptible to damage. Similar trends were observed by Deslauriers _et al_ (1988). They used fluorescein diacetate as a means of identifying and sorting viable _B. napus_ microspores: 0.057 and 0.45% of microspores underwent embryogenesis when selected at 1 and 3 days after culture initiation, respectively. Physical damage due to sheath flow parameters likely limited efficiency of embryo regeneration since the numbers of embryos originating from microspores exposed to 0.85% saline solution for 2 h did not differ significantly from non-exposed controls (data not shown).

We were not able to sort embryogenic populations older than 3 days because of the maximum specimen size limit imposed by the 76 µm diameter aperture. Not only is the aperture size a limiting factor (maximum aperture size commercially available is about 200 µm) but the mechanism of cell sorting using the microdroplet technique would also limit the maximum specimen size to be sorted. A large sized aperture and an alternative sorting strategy, such as continuous stream technique, would need to be adapted. _Brassica_ proembryos, older than 4 days, could then be sorted by size only. This approach would be useful to identify embryos with certain desirable characteristics, such as fatty acid composition, when appropriate fluorescence markers are developed.

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